

Differential release of proteins from bovine fat globule membrane

MALIN, E. L., and BASCH, J. J. 1990. Differential release of proteins from bovine fat globule membrane. *Biochem. Cell Biol.* **68**: 899-902.

Alkaline phosphatase and 5'-nucleotidase are covalently linked to phosphatidylinositol in bovine fat globule membrane, as demonstrated by their release following treatment with phospholipase C specific for phosphatidylinositol. The failure of this treatment to liberate phosphodiesterase I may indicate that it has a variant linkage resistant to release. In a test of exposure at the membrane surface, alkaline phosphatase and phosphodiesterase I, but not 5'-nucleotidase, were released from fat globule membrane by treatment with proteinase K. These apparent differences in accessibilities of membrane surface proteins suggest that attachment to phosphatidylinositol does not necessarily impart greater exposure to proteins with which it is linked.

Key words: alkaline phosphatase, 5'-nucleotidase, phosphodiesterase I, phosphatidylinositol, phospholipase C, fat globule membrane.

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La phosphatase alcaline et la 5'-nucléotidase sont liées par une liaison covalente au phosphatidylinositol au niveau de la membrane de gouttelette lipidique bovine, comme le démontre leur libération après traitement à la phospholipase C spécifique du phosphatidylinositol. Par contre la phosphodiesterase I n'est pas libérée; ceci peut indiquer qu'il existe une liaison modifiée entre la phosphodiesterase I et le phosphatidylinositol, résistante au traitement de la phospholipase C. Lorsque la membrane des globules lipidiques est traitée avec la protéinase K, la phosphatase alcaline et la phosphodiesterase I sont libérées, mais non la 5'-nucléotidase. Ces différences apparentes au niveau de l'accessibilité des protéines de surface membranaire suggèrent que les liaisons avec le phosphatidylinositol n'engendrent pas obligatoirement une meilleure exposition de ces protéines.

Mots clés : phosphatase alcaline, 5'-nucléotidase, phosphodiesterase I, phosphatidylinositol, phospholipase C, membrane de gouttelette lipidique bovine.

Introduction

The discovery that many plasma membrane proteins are covalently attached to PI of the lipid bilayer by a glycan linker (Low 1987) has prompted an increased focus on the details of membrane structure. When these PI-G tailed proteins (Berger *et al.* 1987) are released by a PI-PLC, the glycan-inositol moiety remains attached to the protein. The functional variety of known PI-G tailed proteins (Low 1987; Low and Saltiel 1988) suggests that many spatial orientations could be adopted by PI-linked proteins, since structural details of the PI-G linkages (Low and Saltiel 1988; Ferguson *et al.* 1988; Schmitz *et al.* 1987) indicate prospects for stable anchoring, flexibility, translational motion, and freedom to "float" beyond the membrane surface.

To explore the range of spatial orientations and exposures available to PI-G linked proteins, we investigated proteins of FGM that surrounds lipid droplets during secretion from the lactating mammary cell. FGM is composed of plasma membrane (Patton and Keenan 1975) plus an inner layer of unknown origin (Freudenstein *et al.* 1979) and is a readily prepared model membrane. Among the proteins in bovine

FGM (Patton and Keenan 1975; Dapper *et al.* 1987) that are reported to be PI-G linked in other membranes are ALP, 5'-ND, and PD I (Low 1987; Low and Saltiel 1988). FGM was treated with PI-PLC to determine if these enzymes are released and, therefore, PI-G linked. To assess the external accessibility of each enzyme, FGM was treated with PK, since previous studies with placental ALP have shown that it can be removed from the membrane by certain proteases at single, specific sites located at varying distances from the C-terminus (Jemmerson and Stigbrand 1984; Jemmerson *et al.* 1985; Thompson *et al.* 1987).

Materials and methods**Materials**

PI-PLC purified from *Bacillus thuringiensis* was the gift of Dr. Sidney Udenfriend (Roche Institute of Molecular Biology, Nutley, NJ). All enzymes, proteins, protease inhibitors, substrates, and buffers were products of Sigma Chemical Co.² (St. Louis, MO), except for low molecular weight standards (Pharmacia, Piscataway, NJ), dimethyl sulfoxide (Gold Label grade; Aldrich Chemical Co., Milwaukee, WI), Schiff reagent (Fisher Scientific, Malvern, PA), bicinchoninic acid protein assay reagent (Pierce Chemical Co., Rockford, IL), and silver stain (Bio-Rad, Richmond, CA). FGM was prepared from washed raw cream by extracting acylglycerols of the fat droplets with dimethyl sulfoxide using an adaptation of the method of Dapper *et al.* (1987). Centrifugation at 90 000 × g and 2°C yielded pellets of FGM.

²Reference to brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

ABBREVIATIONS: PI, phosphatidylinositol; PI-G, PI-glycan; PI-PLC, phospholipase C specific for PI; FGM, fat globule membrane; ALP, alkaline phosphatase; 5'-ND, 5'-nucleotidase; PD I, phosphodiesterase I; PK, proteinase K; PMSF, phenylmethylsulfonyl fluoride; XO, xanthine oxidase; ACP, acid phosphatase; SDS-PAGE, sodium dodecyl sulfate - polyacrylamide gel electrophoresis.

¹Author to whom all correspondence should be addressed.

TABLE 1. Release of enzymes from bovine fat globule membrane

	Activity, total units $\times 10^a$		
	ALP	5'-ND	PD I
FGM	22	9.7	11
Control, no enzymes			
Supernatant	0.2	0.1	0.1
Pellet	9.6	8.5	9.9
PI-PLC treatment			
Supernatant	11	3.2	0.2
Pellet	7.2	7.7	9.5
PK treatment			
Supernatant	3.7	0.2	6.0
Pellet	5.7	6.9	3.9

^aAverages of 2-29 assays (SEM = 0.08). One unit of activity equals 1 mmol product/min under assay conditions specified in the text.

PI-PLC treatment

FGM samples, 8.8 mg/mL (based on solids content) in 0.05 M Tris (pH 7.5), were incubated for 60 min at 37°C with PI-PLC at 5 U/mL of buffer-FGM mixture; 3.0 μ M leupeptin and pepstatin and 30 μ M PMSF (final concentrations) were added to inhibit a small amount of proteolytic activity in PI-PLC and any endogenous membrane proteases. Identical samples incubated without PI-PLC served as controls. After incubation, samples were centrifuged at 137 000 $\times g$ for 1 h at 2°C. Pellets were resuspended by homogenization with buffer volumes equivalent to those of supernatants removed. Supernatants and pellets were assayed for ALP, 5'-ND, PD I, and protein concentration. Assays for the inner membrane enzyme XO and for ACP, which is frequently membrane associated, were also conducted.

PK treatment

Similar FGM samples (as above) in 0.05 M Tris - 1 mM Ca²⁺ (pH 8.0) were incubated with PK (1% w/w of the protein content of FGM samples) for 30 min at 37°C. The reaction was stopped by cooling the incubation tubes to 0°C and adding PMSF (final molar concentration = 100 \times that of PK) and leupeptin and pepstatin (final molar concentrations = 10 \times that of PK) to inhibit the action of PK and endogenous proteases. After removal of supernatants, pellets were washed with buffer, recentrifuged, and resuspended with buffer volumes equal to those of the supernatants. Control samples were incubated without PK. After centrifuging as above, supernatants and pellets were assayed for ALP, 5'-ND, PD I, ACP, XO, and protein concentration.

Additional studies with PK were conducted on ALP from bovine liver and 5'-ND and PD I from *Crotalus adamanteus* venom to estimate their susceptibility to PK proteolysis. The amount of PK used was 1% (w/w) of the weight of the protein sample. After a 30-min incubation at 37°C, sample tubes were cooled to 0°C and inhibitors were added to final concentrations 100 \times (PMSF) and 10 \times (leupeptin and pepstatin) that of the PK concentration in each sample, as indicated above. Activity of each enzyme was assayed before and after PK treatment. ALP and PD I were also assayed using α -naphthol phosphate and α -naphthylthymidine 5'-phosphate.

Assays

Assays were conducted at ambient temperature unless otherwise indicated. ALP activity was determined by following at 405 nm the release of *p*-nitrophenol from *p*-nitrophenyl phosphate at pH 10.25 (McComb and Bowers 1972). Activity of 5'-ND was determined at pH 7.4 and 265 nm with a modification of the method of Ipatia (1967). The assay mixture included β -glycerophosphate to inhibit the action of ALP on 5'-AMP (Belfield and Goldberg 1968). PD I activity at pH 8.9 was followed at 400 nm using *p*-nitrophenylthymidine 5'-phosphate as substrate (Razzell and Khorana 1959). Activity of ACP was assayed by monitoring *p*-nitrophenol

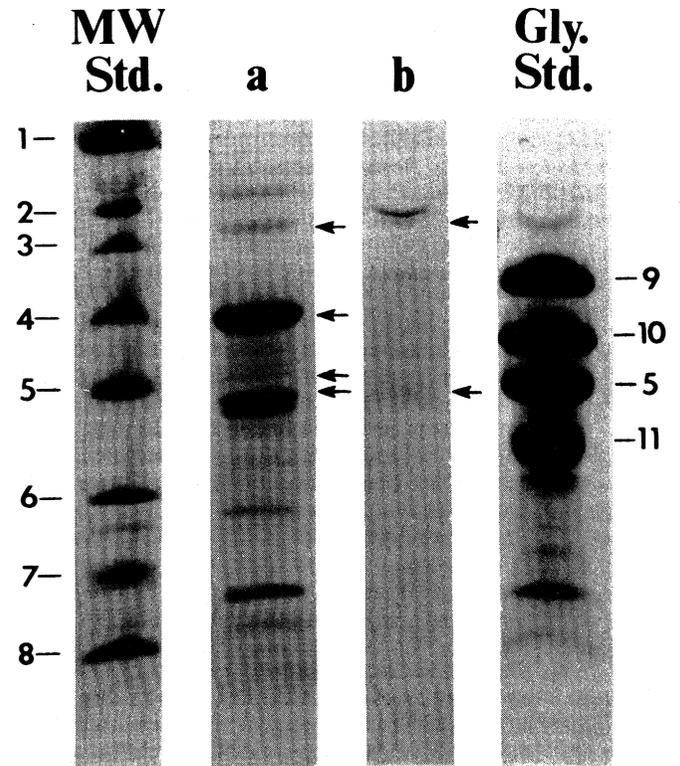


FIG. 1. SDS-PAGE of supernatants from PI-PLC treatment of FGM. Gels were stained with Coomassie blue. Arrows mark bands which stained for carbohydrate on identical gels. Left lane, molecular weight standards (MW Std.); lane a, supernatant from PI-PLC treatment; lane b, control supernatant from FGM mixed with buffer; right lane, carbohydrate standards (Gly. Std.). 1, myosin; 2, β -galactosidase; 3, phosphorylase b; 4, bovine serum albumin; 5, ovalbumin; 6, carbonic anhydrase; 7, soybean trypsin inhibitor; 8, α -lactalbumin; 9, lactoferrin; 10, catalase; 11, riboflavin binding protein.

concentration after a 30-min incubation with *p*-nitrophenyl phosphate in acetate buffer (pH 5.5) at 37°C; pH was increased to 10 for 410-nm readings (Boman 1955). XO activity was determined at pH 8.3 by following the oxidation of xanthine to uric acid at 295 nm (Massey *et al.* 1969). Protein concentration was determined with the bicinchoninic acid reagent using bovine liver ALP as the standard.

Electrophoresis

SDS-PAGE was performed with the PhastSystem (Pharmacia, Piscataway, NJ) using 8-25% gradient gels. Gels were stained with Coomassie blue R250 or with the Schiff reagent for glycoproteins (Kapitany and Zebrowski 1973). Combined silver and Coomassie blue stains (Dzandu *et al.* 1984) were occasionally used to verify results of Coomassie blue staining alone. Molecular weights of protein bands were estimated from plots of log molecular weight versus mobility.

Results

Release by PI-PLC

Assays demonstrated substantial release of ALP and 5'-ND activities by treatment with PI-PLC accompanied by corresponding loss of activities from pellets, indicating that these enzymes are PI-G linked in FGM (Table 1). However, no increase was observed for PD I activity, although small amounts appeared to "leak" out (see below). The significance of the PD I data is considered later (see Discussion). About

TABLE 2. Effect of proteinase K on activity of membrane enzymes

	Specific activity, units/mg ^a		
	ALP	5'-ND	PD I
Before treatment	7.9 ^b	2.2	10 ^c
After treatment	3.6 ^d	—	0.63 ^e
	4.2 ^b	2.7	12 ^c
	0.02 ^d	—	0.0 ^e

^aALP from bovine liver; 5'-ND and PD I from *C. adamanteus* venom; averages of duplicate assays.

^bSubstrate = *p*-nitrophenyl phosphate.

^cSubstrate = *p*-nitrophenylthymidine 5'-phosphate.

^dSubstrate = α -naphthol phosphate.

^eSubstrate = α -naphthylthymidine 5'-phosphate.

6% of the FGM protein concentration appeared in control supernatants from FGM incubated with buffer; protein concentrations of supernatants from PI-PLC treatment were one third greater. XO and ACP activities in PI-PLC supernatants were not enhanced, indicating absence of PI-G linkage in FGM.

Four major and several minor protein bands were observed after SDS-PAGE of supernatants from PI-PLC treatment (Fig. 1). Because many are glycoproteins, true molecular weights may differ from those estimated from plots of log molecular weights versus mobility. The strongest glycoprotein band, 68 000, could contain subunits of ALP and 5'-ND, as well as butyrophilin, an interpretation based on mobilities that we observed for ALP from several bovine tissues and 5'-ND from *C. adamanteus* venom. Values of 65 000 to 73 000 for 5'-ND (Thompson *et al.* 1987; Dornand *et al.* 1978) and 67 000 for butyrophilin (Heid *et al.* 1983) have been reported. The upper arrows in Fig. 1, lanes *a* and *b*, indicate "leaked" glycoprotein bands of 115 000 in both supernatants. These bands may be PD I, since they correspond to values of PD I observed for *C. adamanteus* venom and reported for rat intestine (Nakabayashi and Ikezawa 1986).

FGM is more particulate than most plasma membranes and is known to be "leaky." Assays (Table 1) and electrophoresis (Fig. 1) showed that proteins appeared in control supernatants in the absence of PI-PLC when FGM was mixed with buffer and incubated at 37°C. Such leakage occurred even when mixing of FGM and incubation buffer was avoided. Staining with Coomassie blue and Schiff reagent indicated that all components which leaked into the control supernatant also appeared in the PI-PLC supernatant. PI-PLC treatment specifically released ALP and 5'-ND into the treated supernatant, thereby increasing the content of these enzymes over that released by leakage, as indicated in Table 1.

Release by PK

PK is a serine endopeptidase, homologous to bacterial subtilisins and with little specificity (Betzel *et al.* 1988), which can clip off proteins exposed at the membrane surface. ALP and PD I were both released by treatment with PK (Table 1), although activity of the released ALP was less than that released by PI-PLC. As discussed below, the shortened ALP chain was still active with *p*-nitrophenyl phosphate as substrate, but loss of part of the carboxyl terminus may have destabilized the active site, leading to decreased activity. Release of PD I indicated that it is highly exposed at the membrane surface. In contrast, 5'-ND was unaffected by PK, suggesting that it is not exposed to the same extent as

ALP or PD I. Table 1 indicates that substantial 5'-ND activity remained with the pellet after PK treatment. Activities of XO and ACP were not increased in supernates from PK.

To confirm these results, the effects of PK treatment on size and activity of ALP (bovine liver) and 5'-ND and PD I (both from *C. adamanteus* venom) were investigated. Specific activity of bovine liver ALP decreased 52%, but activities of venom 5'-ND or PD I remained the same (Table 2). Both ALP and PD I still functioned with *p*-nitrophenyl substrates, but activity with the larger α -naphthyl substrates was lost. These results indicate that 5'-ND is susceptible to attack by PK, under conditions similar to those used for PK treatment of FGM, but does not lose activity. In FGM, accessibility of the 5'-ND sites that are normally susceptible to attack by PK may be partially or completely blocked.

Addition of inhibitors after incubation with PK prevented further proteolysis and loss of activity in assays for several days, but PK-released proteins seemed unable to survive electrophoresis. Faint bands of shortened ALP and PD I were seen in supernates of PK-treated FGM after SDS-PAGE with silver - Coomassie blue or carbohydrate staining. ALP and 5'-ND each lost about 12 000 to 13 000 of their molecular weight and PD I about 26 000, approximately one fifth of each enzyme. However, losses of this magnitude would not necessarily occur when these proteins are membrane bound.

Discussion

Each major FGM surface enzyme investigated appears to have different topological characteristics. ALP was released by both PI-PLC and PK, PD I was unexpectedly not susceptible to the former, and 5'-ND was not accessible to the latter, as previously demonstrated when human placenta was treated with PK (Thompson *et al.* 1987). The substantial release of ALP and 5'-ND by PI-PLC, compared with controls, confirms that these enzymes are linked to PI in the FGM lipid bilayer, as in other membranes (Low 1987; Low and Saltiel 1988). As expected, XO and ACP were not susceptible to the actions of PI-PLC or PK.

Although the failure of PI-PLC to release PD I suggests that there is no PI-G link to PD I in FGM, this conclusion may be unwarranted. Earlier work showed that PD I was not liberated by PI-PLC in rat pancreas and liver, although PI-G linkage was present in other rat organs (Nakabayashi and Ikezawa 1986). Two interpretations may explain these observations. The PI-G link may not be expressed for some isozymes of PD I, being replaced by polar or electrostatic interactions between enzyme and lipid bilayer to provide stable anchoring. A more probable explanation is that the PI-G link possesses a variant inositol moiety resistant to PI-PLC, as has been discovered for acetylcholinesterase (Roberts *et al.* 1987) and DAF (Davitz *et al.* 1986) of human erythrocyte membrane.

Although both ALP and 5'-ND are PI-G linked, the inability of PK to release 5'-ND indicates that its covalent linkage to PI does not guarantee an exposed orientation at the membrane surface. Additional structural features evidently protect 5'-ND from PK attack on FGM. The enzyme may be embedded in the membrane surface with only the carboxyl terminal and its PI-G link exposed, perhaps as a loop, where it may be reached by PI-PLC. The orientation of 5'-ND may hide domains of the enzyme that were susceptible to PK attack in solutions of free 5'-ND. The possibility that 5'-ND is partially removed by PK and

then degraded can be considered, but this rationale would contradict the work of Thompson *et al.* (1987) who used PK to remove potentially contaminating ALP from the surface of placental membrane before isolating 5'-ND.

In contrast, ALP appears to be almost completely externalized, providing access to the action of PI-PLC, as well as to proteolytic attack by PK (Thompson *et al.* 1987), trypsin (Jemmerson and Stigbrand 1984), and bromelain (Jemmerson *et al.* 1985). The PI-G linkage seems to allow ALP to lie partly or completely above the membrane surface, perhaps "floating" in intercellular space, in analogy with ALP of *Escherichia coli* in which the enzyme is not PI-G linked but localized in the periplasm (Reid and Wilson 1971). The PI-G link may help maintain ALP function at sites external to the cell membrane. A second alternative is that ALP is an amphipathic protein which interacts with the polar head groups of membrane phospholipids. However, the enhanced lateral diffusion reported for ALP (Noda *et al.* 1987) indicates that this is less likely. Purification and characterization studies now underway in this laboratory on FGM ALP released both by PI-PLC and by PK should reveal how much of the enzyme chain is externalized.

The inability of 5'-ND to be released by PK is strong evidence that a PI-G link is not the sole determinant for the topology of membrane-bound enzymes. Functional requirements may be the essential forces dictating degree of exposure for membrane surface proteins; the PI-G link may only be an additional stabilizing force for a functionally required orientation. A more complete understanding of differences among ALP, 5'-ND, and PD I orientations and accessibilities in FGM may eventually clarify the role of the PI-G link, as well as explain the functions of these enzymes in this and other membranes.

Acknowledgements

We thank Dr. Sidney Udenfriend and Dr. Martin Low for helpful discussions and gifts of PI-PLC. Technical support was ably provided by Brien C. Sullivan, Nya Patrinos, and Sydney Shrader.

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